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Application of electrospun gelatin nanofibers in tissue engineering

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ABSTRACT

The aim of this work was to prepare gelatin nanofibers and investigate their medical applications such as nanofibrous scaffolding for skin regeneration, and skin wound dressing in tissue engineering. First, gelatin nanofibers were prepared via electrospinning method. The morphology and diameter of the nanofibers were characterized by scanning electron microscopy (SEM).Based on the mechanical weakness of gelatin nanofibers, the heat treatment process was used, and the effects were investigated via mechanical tests. The results of the stress strain curve demonstrated that the mechanical properties of the nanofibers enhanced after heat treatment.The heat treated electrospun gelatin nanofibers were applied as scaffold, and human foreskin fibroblast cells (HFFs) were cultured on the scaffold and characterized by SEM. The results showed that the HFFs were completely spread on the surface of the scaffold. In addition, the toxicity of the scaffold was investigated using MTT assay. The scaffold exhibited desired biocompatibility and no toxicity after 24 and 48 hours in culture. In the other ways, the gelatin nanofibers were investigated as a wound dressing in vivo. The certain amount of *Myrtus Communis* essential oil was loaded in gelatin nanofibers and was used as skin care dressing in vivo. The results of this study indicated no positive effects of *Myrtus Communis* essential oil gelatin nanofibers (*MEG)* on the healing of the wound.

Keywords: *electrospinning, nanofibers, gelatin, wound healing.*

1. INTRODUCTION

Nanofibers, the sub-microfibers, have the attractive interest because of their unique properties (i.e., low weight, high porosity and high surface area to volume ratio) [1-8].These properties improve growth and enhance the proliferation rate of seeded cells by facilitating diffusion of oxygen and nutrients throughout the scaffolds [9]. Considerable attention has been paid for the application of nanofibers in tissue engineering as scaffold due to their abilities in mimicking the native extracellular matrix (ECM) [10].

During the past years, a number of methods have been developed for the fabrication of nanofibers such as phase separation; melt blowing, self-assembly, forcespinning and electrospinning [2, 8]. Among these, electrospinning is the most common method in the production of nanofibers [1, 11]. Electrospinning technique was used to fabricate gelatin nanofibers. A basic electrospinning apparatus consists of a high voltage power supply, a reservoir for polymer solutions and a conductive collector and a nozzle. Basically, the potential difference between the syringe nozzle and the collector leads to stretch of solution and formation of fibers. The syringe is pushed by a syringe pump for continuous production of fibers. To establish potential difference, a positive electrode is attached to the syringe tip and a negative electrode to the conductive collector. A high voltage electrostatic field is used to charge a viscous fluid and stretch into the collector. Then, after a stretch of the solution to collector, charged solvent evaporates and solid fibers remain and deposit at the cylindrical collector [1].

Regeneration of damaged tissue via nanofibers requires designing an ECM-like scaffold with high surface area to volume ratio and high porosity for promoting homogenous cell attachment, proliferation and mineralization throughout the scaffold [12]. In addition, biocompatible and biodegradable scaffolds designed by natural polymers (i.e., collagen, gelatin) may provide ECM-like structure, better environment for developing seeded cells in comparison with synthetic polymers. ECM structure contains several materials and biopolymers (i.e., collagen, fibronectin and laminin, etc.) [13].

Gelatin, a biodegradable, biocompatible and edible polymer, is caused by collagen hydrolysis, the most abundant protein in the ECM [2]. Gelatin in comparison with the other biopolymers, e.g., collagen, may provide a better environment for cell attachment, growth and proliferation. Fibronectin (FN), an abundant soluble constituent of plasma, has a notably wide variety of functional sites besides binding to cell surfaces through integrin and collagen, etc. For instance, the collagen-binding domain in FN binds effectively to gelatin (denatured collagen) even more than

native collagen [14]. Therefore, based on the unique properties of gelatin, it could be a notable candidate using as a cell scaffold or skin dressing in the field of tissue engineering.

In traditional medicine, *Myrtle* (Myrtus communis L., Myrtaceae) is considered as a medicinal herb, and several components such as *polyphenols*, *myrtucommulone*, and *limonene* have been extracted from *Myrtle*. The clinical and experimental studies propose an extensive spectrum of therapeutic and pharmacological effects of *Myrtle* such as anticancer, antifungal, antiviral activity [15], which may have health benefits due to anti-inflammatory, antibacterial, antiseptic and anti-congestive properties [16].

In this study, we prepared gelatin nanofibers using electrospinning method. Then, the gelatin nanofibers were investigated as scaffolding in tissue engineering. In addition, the effects of *MEG* on wound healing were studied in vivo.

2. EXPERIMENTAL SECTION

2.1. Materials. Gelatin and glacial acetic acid (Merck, Germany) were used as base polymer and solvent for producing nanofibers, respectively. Essential oil of *Myrtus Communis* (Registration number: 1228022715:(IRC)) was purchased from local pharmacies. The electrospinning process was done using Electroris (FNM Ltd., Iran, www.fnm.ir).

2.2. Fabrication of gelatin nanofibers. The glacial acetic acid was used as solvent for producing a sample of gelatin nanofibers. The sample of gelatin nanofibers was prepared with defined process parameter (table 1).Syringe pump was set at 1 ml/h for flow rate. The distance between the nozzle and collector was 10 cm and drum was fixed for 200 rpm. The nanofibers images were taken by SEM to evaluate fibers diameter and morphology. Then, mean diameter of 20 fibers was computed by the Image J software (Sun Microsystems, USA).

2.3. Fabrication of *MEG.* Like gelatin nanofibers, the glacial acetic acid was used as solvent for producing a sample of gelatin/ *Myrtus Communis* essential oil (essential oil of *Myrtus Communis*,based on 9-15 mg 1,8 *cincole* in each ml of product, Manufacture: Barij essence pharmaceutical.co -batch no: 90127) nanofibers. The several nanofibers with different solvents and electrospinning parameters were produced. The defined parameters for producing a selected sample of nanofibers were shown in table 2.The concentration of gelatin and *Myrtus Communis* essential oil in defined nanofibers was 20% and 1% (V/V), respectively. The distance between the nozzle and collector was set at 10 cm and drum was fixed at 250 rpm, and flow rate was set at 0.5 ml/h.

2.4. Preparation of culture medium for the maintenance of fibroblast cells. HFFs were cultured on the gelatin nanofibers scaffold. Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) was prepared and kept in cold temperature (4°C). For controlling culture medium acidity, an indicator was used to determine the pH of the medium. A solution of phenol red can be used as a pH indicator. Phenol red color changed from red to yellow after adding to culture medium due to consumption of nutrient and excretion of acidic waste metabolites. Hence, culture medium was replaced with a new one's after changing the color.

2.5. Cell maintenance. To increase cell growth and proliferation, HFFs were kept in the cell culture flask under incubation conditions in the atmosphere controlled chamber (temperature: 37°C,CO_2 concentration: 5%, humidity and other factors similar to the condition of the human body). After changing color, culture medium was replaced with a new one to provide nutrients for living cells. Sulfate water was used to provide both humidity and an antifungal medium in the incubator. The filters were applied in an incubator for exchange CO_2.

2.6. Cell separation. Cell-passage activity was performed to enhance cell proliferation. Based on the results, the highest proliferation rate in long-term cultivation has been seen in the 4^{th} cell passage [17]. Briefly, in order to achieve this goal, in the culture container, the liquid media covering cells were depleted and dissociation reagent, trypsin, was added to the vessels and incubated for about 5 minutes. Then, preserved medium inside the flask was transported to the Falcon tube (50 ml) containing culture medium with 10% FBS, and immediately centrifuge for 5 minutes at 1800 rpm. After aspiration of supernatant, freshly culture media were added to the Falcon tube containing remained cell pellet. Finally, cells on the bottom of the container were detached and prepared for the next stages.

2.7. Cell counting. After detachment of the cells from the base of the Falcon tube, 10 µl of uniform suspension of HFFs was combined with 10 µl of Trypan blue solution (A vital stain for assessing the cell viability via the dye exclusion test). Then, the prepared suspension was placed on the *neobar* slide and was assessed under light microscope. In the Trypan blue viability assay, living cells had white color (unstained), whereas the dye passed through the dead cell membrane and took up the dark blue stain of Trypan blue (stained) [18].

2.8. MTT assay. A sample of gelatin nanofibers, which was treated via heating, was placed in 24-well plate under hood condition. Then, about 2×104 HFFs were counted by *neobar* lam and added to the wells with 1 ml of culture medium and were grown. After 24 and 48 hours in culture, MTT formazan solution was added to the wells and incubated for 4 hours in the dark. Finally, the absorbance of the wells contents was measured at 570 nm using a spectrophotometer.

2.9. SEM images. The gelatin nanofibers were produced by electrospinning, and their images were taken via SEM, after sputtering with gold. The mean diameter of randomly 50 fibers of electrospun gelatin nanofibers using size analysis software was calculated and considered as the mean diameter of the samples.

2.10. Mechanical properties. The stress-strain behavior of gelatin nanofibers was evaluated by means of a mechanical testing machine (STM Series Machine Control and Report Software (CIH 2001 Company)). The thickness of electrospun gelatin nanofibers was measured by means of a digital micrometer. The test was

performed on the rectangular section type and samples with 30 mm length, 10 mm width and 0.13 mm thickness at the deformation rate of 10 mm per minute.

2.11. In vivo studies. The wound healing test was performed according to Moreira et al experiments [19]. Briefly, mice were anesthetized by intraperitoneally injecting a mixture of ketamine 100 mg/kg and xylazine 10 mg/kg, which was diluted in 100 µl of saline solution. Then, a hair removal machine was used to remove their hairs from the mice dorsum and prepared the surgical site with 70% alcohol. The dorsal skin cranially and caudally was folded and raised by the index fingers and thumbs. Then, the animal in a lateral position was placed and pressed down the biopsy punch with 5 mm diameter to remove the two skin layers and created excisional wounds.

After surgery, the mice were moved to a warm area and monitor their recovery from anesthesia, and the fully recovered mice return to their routine housing.

2.12.The wound healing test steps. Two types of nanofibers loaded with different percentages of *MEG* (1% and 2%) were prepared by electrospinning method.

In the animal test, six mice were placed in each group. One of the two ulcerations of biopsy punch in each mouse was selected as the control and experiment. The right wounds were determined as a test and the left wounds as the control, and the wounds were covered with a one centimeter square sample of nanofibers containing the extract and the commercial wound dressing (Beta), respectively. To determine the wound healing process, the dressing was changed every day, and the area of the wounds was measured at 1, 2, 3, 4, 5, 6, 7, 8 and 10 days.

2.13. Lesion closure monitoring after punching. To measure the larger *(A)* and minor *(B)* diameters of the wounds and control their area via a caliper, the following formula *(1)* based on(Circle area= πr^2) was applied[19].

(1) (diameter A/2) x (diameter B/2) x π.

The percentage of lesion healing is defined as $F/O \times 100\%$, where O is the original wound area and F is the wound area after a fixed time interval [19].

2.14.Statistical analysis. Data were collected from the six samples and were expressed as mean ± standard deviation (S.D.). Statistical analysis was performed with Student's *t*-test and significance was determined at $p < 0.05$.

3. RESULTS SECTION

3.1. Nanofibers morphology. SEM image taken from the morphology of electrospun gelatin nanofibers indicated that nanofibers were approximately smooth and defect-free. In addition, the mean diameter of nanofibers was 97 nm and most of nanofibers diameter were less than 100 nm. The nanofibers diameter has been often uniform along with an individual nanofiber as well (as shown in Fig. 1).

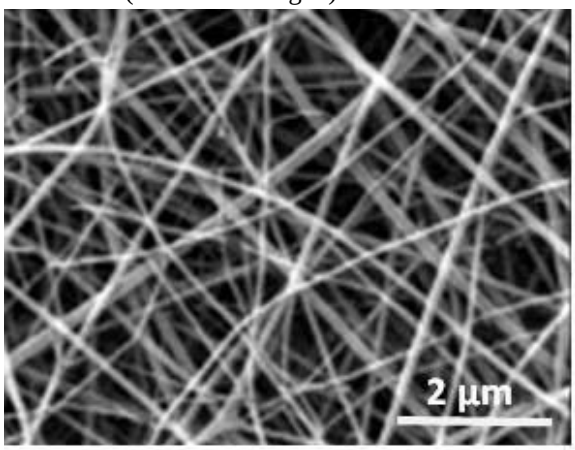


Figure 1. SEM image of electrospun gelatin.

3.2. Nanofibers mechanical properties. The tensile stress–strain curves of the electrospun nanofiber scaffolds are presented in Fig. 2. Curves of 2a, b and c are related to electrospun gelatin nanofibers heated at room temperature (untreated), 40°C and 80°C for 30 min (treated), respectively. The measured Young's module and maximal stress of the electrospun nanofibers increased as temperature enhanced. The difference between the tensile strength of heat treated and untreated nanofibers could be due to the heat-induced crosslinking [20]. Therefore, greater mechanical properties and higher resistance against breaking was one of the effects of heat treating on gelatin nanofibers [21]. It is worth noting that the stress–strain curves for the gelatin scaffold are repeatedly zig–zag that's probably due to inter-fiber slipping or small-scale tensile failure in subsets of the scaffolds fibers.

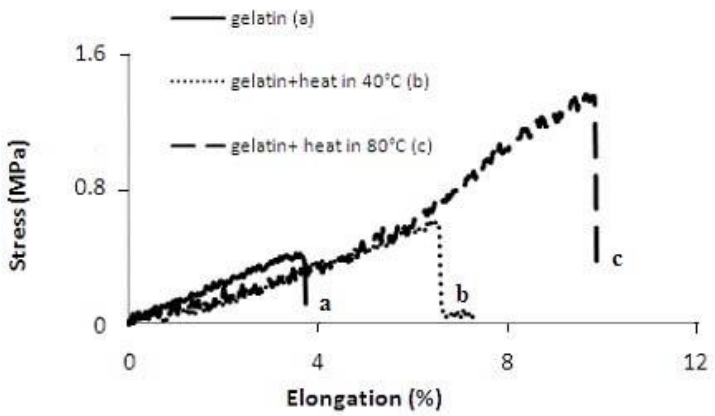


Figure 2. Tensile stress–strain curves of electrospun gelatin nanofibers: (a) gelatin nanofibers without heat; (b) gelatin nanofibers with heat in 40°C; (c) gelatin nanofibers with heat in 80°C.

3.3. Toxicity assay. MTT assay was used to determine viability of the cells cultured on the nanofiber scaffold. This colorimetric assay is based on the ability of living cell enzymes; mitochondrial succinate dehydrogenase reduces yellow tetrazolium dye [22]. MTT causes a yellowish solution which passes from cell walls, enters into the mitochondria and reduces formazan to an insoluble dark purple using aforementioned enzyme. The intensity of formazan as colorimetric measurement was used via spectrophotometer at 570 nm. The intensities of optical absorption have a linear and a direct relationship with the living cells. In other

words, reduction of tetrazolium salt depends on the number of living cells. It means that intensities of optical absorption increase or decrease as the number of living cells increase or decrease. This matter is due to increase or decrease in the reduction of tetrazolium salt, MTT, by living cells to blue formazan product; therefore, living and growth rate of cells can be indirectly measured via MTT assay [23]. The results of MTT assay demonstrated that the gelatin nanofibers may be a good candidate as a base material for cell scaffolding. The spectrophotometric curves indicated higher percentage of absorbance in the experimental group (seeding cells on the scaffold) than the control group (cell pellet culture). The results confirmed that the biocompatible gelatin scaffold provides better growth conditions for seeded cells in the experimental group than the control group (as shown in Fig. 3).

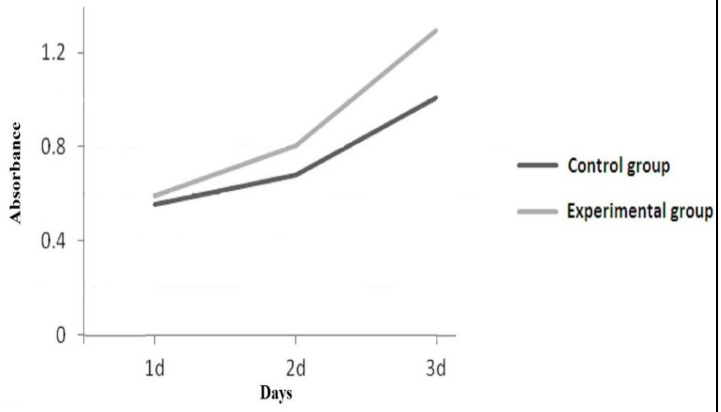


Figure 3. The spectrophotometer absorbance in the experimental group (scaffold with seeded HFFs) and control group (HFFs without nanofiber scaffolds) at first, second and third days.

3.4. Cell morphology via SEM image. The morphology of the seeded HFFs in the gelatin nanofibers scaffold (24 and 48 hours after seeding) was investigated by SEM images (as shown in Fig.4). In this experiment, cell attachment, pseudopods emerging, networking and spreading steps were considered to evaluate biocompatibility of scaffolds. The morphology of the cultured HFFs was demonstrated via SEM images for 24 and 48 hours after seeding. As shown in Fig. 4, two days after cell culturing on the scaffold, pseudopods were observed on the scaffold using SEM images. HFFs were completely spread on the surface of the scaffold which indicates the desired biocompatibility of gelatin nanofibers.

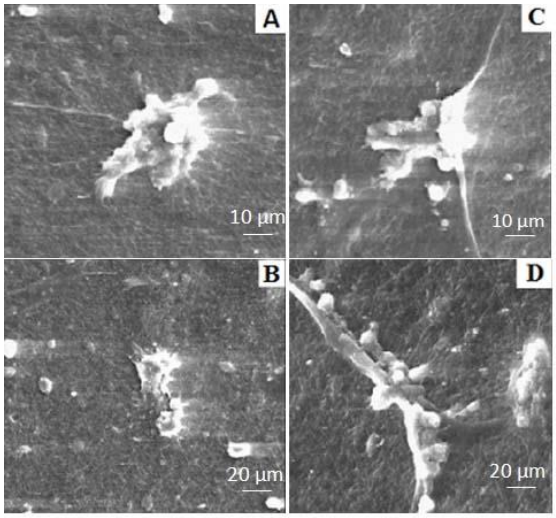


Figure 4. SEM images of HFFs seeded on the gelatin nanofibers scaffolds after (A, B) 24 h and (C, D) 48 h.

3.5. In vivo experiment. The changes in wound areas at different healing times using *MEG*, and a commercial wound dressing (Beta, control) was shown in Fig. 5. The wound areas decreased gradually and reached about 7% after 10 days in the control group, while via the wound dressings, the lesion area reached about 21% (Fig.6). Therefore, the effects of the *MEG* on healing process were worse than commercial wound dressing ($p < 0.05$)**.**

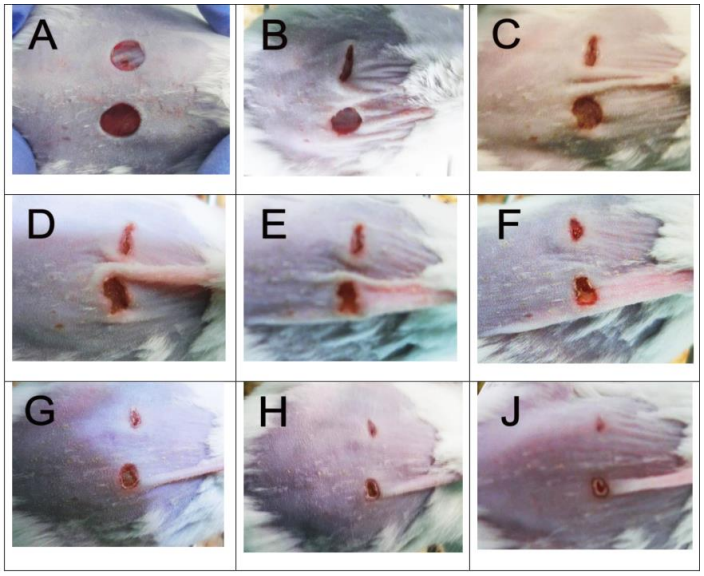
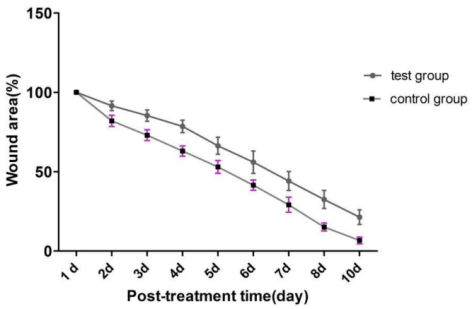


Figure 5. The sample of process of wound healing within 10 days in vivo(A=1d), (B=2d), (C=3d), (D=4d), (E=5d), (F=6d), (G=7d), (H=8d), (J=10d).



Groups / Day (d)	Test group		Control group	
	Mean	SD	Mean	SD
First d	100	0	100	0
Second d	91.50	3.016	82	3.58
Third d	85.33	3.559	73	3.40
Fourth d	78.50	3.885	63	3.22
Fifth d	66.33	5.354	53	4.04
Sixth d	56	7.042	41.5	3.27
Seventh d	44.16	5.98	29.16	4.79
Eighth d	32.5	5.683	15.16	2.48
Tenth d	21.33	4.633	6.66	2.16

Figure 6. The changes in wound areas at different healing times using *MEG* (test group), and a commercial wound dressing (Beta, control group).

Based on the wound healing process, the electrospun *MEG* membrane showed good and immediate adherence to the wet wound surface, but based on our research finding, it had no positive effects on the healing process of skin lesions in mice. Due to the clarity of the delay in wound healing in the experimental group, the histological test was not performed.

Table 1. Data of electrospinning setting for producing gelatin nanofibers

Gelatin concentration (% Wt)	AcOH concentration (% V/V)	Applied voltage (KV)	Temperature (^{o} C)	Mean Diameter (nm)
14	35	11	35	97

Table 2. Data of electrospinning setting for producing *MEG*

Gelatin concentration (% Wt)	Herbal extract (% V/V)	AcOH concentration (% V/V)	Applied voltage (KV)	Temperature (^{o} C)	Mean Diameter (nm)
20	1	60	12	28	120

4. CONCLUSIONS

The natural, biodegradable and biocompatible polymers such as gelatin have attracted many interests for the application as a scaffold in tissue engineering. In this study, the gelatin nanofibers with a mean diameter of 97 nm were selected as a scaffold. Based on the results, desired growth and proliferation of cells on the scaffold was investigated. The results indicated that the HFFs were completely spread on the nanofibers surface, and pseudopods were emerged on gelatin nanofibers for 24 and 48 hours after seeding on the nanofibers surface, and gelatin nanofibers scaffold had no toxic effects on the seeded cells. In addition, the mechanical properties of gelatin nanofibers scaffolds improved due to heat-induced crosslinking. The electrospun nanofiber *MEG* membrane was evaluated as a wound dressing. However, in this study, the *MEG* indicated no positive effect on healing of the wound closure, but apparently neither toxicity nor permeability to exogenous microorganism was observed with the *MEG* dressing.

In this study, only two concentrations of the *MEG* (one and two percent) were applied in the experimental process. It is recommended that the other concentrations of *MEG* could be tested in wound dressing process for evaluation of its role in improving the skin lesion. Also, it suggested that the other natural or synthetic polymers or mixtures can also be used as a nanofibers base of wound coating.

5. REFERENCES

[1] M. Naghibzadeh, *Trends Biomater Artif Organs*, 26**, 2012**.
[2] M. Naghibzadeh and M. Adabi, *Fiber Polym*, 15, 767, **2014**.
[3] N. Ketabchi, M. Naghibzadeh, M. Adabi, S. S. Esnaashari, and R. Faridi- Majidi, *Neural Comput Appl*, 28, 3131, **2017**4. M.
[4] Adabi, R. Saber, R. Faridi-Majidi, and F. Faridbod, *Mater Sci Eng C*, 48, 673, **2015**.
[5] M. A. Karimi, P. Pourhakkak, M. Adabi, S. Firoozi, M. Adabi, and M. Naghibzadeh, *e-Polymers*, 15, 127, **2015**.
[6] M. Adabi, R. Saber, M. Naghibzadeh, F. Faridbod, and R. Faridi-Majidi, *RSC Adv*, 5, 81243, **2015**.
[7] S. Hosseinzadeh, M. Mahmoudifard, F. Mohamadyar-Toupkanlou, M. Dodel, A. Hajarizadeh, M. Adabi, and M. Soleimani, *Bioprocess Biosyst Eng*, 39, 1163, **2016**.
[8] M. Naghibzadeh, M. Adabi, H. R. Rahmani, M. Mirali, and M. Adabi, *Adv Polym Tech*, **2017** (DOI: 10.1002/adv.21817).
[9] H. Fouad, T. Elsarnagawy, F. N. Almajhdi, and K. A. Khalil, *Int J Electrochem Sci*, 8, 2293, **2013**.
[10] S. Srouji, T. Kizhner, E. Suss-Tobi, E. Livne, and E. Zussman, *J Mater Sci Mater Med*, 19, 1249, **2008**.
[11] H. Samadian, S. S. Zakariaee, M. Adabi, H. Mobasheri, M. Azami, and R. Faridi-Majidi, *RSC Adv*, 6, 111908, **2016**.
[12] C. E. Pedraza, B. Marelli, F. Chicatun, M. D. McKee, and S. N. Nazhat, *Tissue Eng Part A*, 16, 781, **2009**.
[13] D. E. Ingber, J. A. Madri, and J. Folkman, *In Vitro Cell Dev Biol*, 23, 387, **1987**.
[14] R. Pankov and K. M. Yamada, *J Cell Sci*, 115, 3861, **2002**.
[15] G. H. R. Hasanzadeh, R. Ghorbani, L. Akhavan, and Z. Nori, *J Iran Anat Sci*, 1, 21, **2003**.
[16] A. Rezaie, D. Mohajeri, B. Khamene, M. Nazeri, R. Shishehgar, and S. Zakhireh, *Curr Res J Biol Sci*, 4, 176, **2012**.
[17] W. Pradel, R. Mai, T. Gedrange, and G. Lauer, *J Physiol Pharmacol*, 59, 47,**2008**.
[18] W. Strober, *Curr Protoc Immunol*, A3, **2001**.
[19] C. F. Moreira, P. Cassini-Vieira, M. F. da Silva, and L. S. Barcelos, "Skin wound healing model-excisional wounding and assessment of lesion area", Bio-protocol, **2015**.
[20] R. Mincheva, N. Manolova, D. Paneva, and I. Rashkov, *J Bioact Compat Polym*, 20, 419, **2005**.
[21] Y. Zhu, M. F. Leong, W. F. Ong, M. B. Chan-Park, and K. S. Chian, *Biomaterials*, 28, 861, **2007**.
[22] A. A. Haroun, A. Gamal-Eldeen, and D. R. K. Harding, *J Mater Sci Mater Med*, 20, 2527, **2009**.
[23] F. Denizot and R. Lang, *J Immunol Methods*, 89, 271, **1986**.

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